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Generating knockout rats by transposon mutagenesis in spermatogonial stem cells

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Abstract

Disrupting genes in the rat on a genome-wide scale will allow the investigation of many biological processes linked to human health. Here we used transposon-mediated mutagenesis to knock out genes in rat spermatogonial stem cells. Given the capacity of the testis to support spermatogenesis from thousands of transplanted, genetically manipulated spermatogonia, this approach paves a way for high-throughput functional genomic studies in the laboratory rat.

Gene targeting in pluripotent embryonic stem cells has been especially successful for generating mice with specifically altered alleles to study disease processes in the whole mouse¹. But cultured stem cells have not been used to modify genes in the germline of other mammalian species fundamental to biomedical research, such as the laboratory rat. Fortunately, advances in growing germline-competent stem cells in tissue culture, including spermatogonia^{2,3} and embryonic stem cells^{4,5}, offer new avenues for genomic manipulations in the rat.

The seminiferous epithelium of the rat testes provides a specialized niche that contains thousands of spermatogonial stem cells, which endow rats with an ability to produce millions of spermatozoa each day during peak reproductive capacity⁶. Rat spermatogonia can be grown

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AUTHOR CONTRIBUTIONS

F.K.H. provided the concept on applications of libraries of transgenic or mutant donor spermatogonial lines, established methods for and executed experiments on *in vitro* spermatogonial culture, *in vitro* plasmid-based gene delivery into spermatogonial stem cells, clonal selection for genetically modified rat spermatogonial stem cell lines *in vitro*, transplantation and germline transmission from genetically modified donor rat spermatogonia using male-sterile recipient rats, analyzed the data, supervised the project and wrote the paper. H.P. analyzed gene expression in mutant rats, genotyped mutant rats and isolated gene trap insertions by splinkerette PCR. K.C. performed rat spermatogonial culture experiments, analyzed gene expression in mutant rats and isolated gene trap insertions by splinkerette PCR. J.S. performed experiments on spermatogonial transplantation and germline transmission from male-sterile rats, genotyped and maintained the colonies of mutant rats. I.G. generated the gene trap Sleeping Beauty transposon and tested its activity in human HeLa cells. J.F. isolated gene trap insertions by splinkerette PCR. Z.Iz. analyzed the data, supervised the project and wrote the paper. Z.Iv. provided the concept of transposon mutagenesis in spermatogonial stem cells, analyzed the data, supervised the project and wrote the paper.

COMPETING FINANCIAL INTERESTS

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in vitro under conditions in which they proliferate and maintain their potential to develop into spermatozoa when transplanted into testes of a compatible host^{2,3}. Moreover, after transduction with lentiviral vectors, freshly isolated donor spermatogonia have been used to produce transgenic rats⁷. Thus, once transplanted into recipient testes, complex genomic libraries in mutant spermatogonial stem cells could theoretically facilitate large-scale production of genetically distinct mutant rats from only a small number of recipient founders (Supplementary Fig. 1).

Insertional mutagenesis using engineered transposable elements has proven to be one of the most productive and versatile strategies for disrupting and manipulating genes on a genome-wide scale in organisms as diverse as plants, worms, flies, fish and mice⁸. Transposable elements became accessible for vertebrate genomics after the creation of Sleeping Beauty, a synthetic transposon generated from defective copies of an ancestral Tc1/mariner-like transposon in fish⁹. Mutagenesis screens in mammals established that transposable elements generate many random mutations in both mouse and rat germinal cells *in vivo*^{10–13}. This had been accomplished by breeding transgenic mice and rats that express the Sleeping Beauty transposase with animals that harbor the Sleeping Beauty transposon^{10–13}. This approach, however, does not permit one to preselect for transposition events that disrupt gene expression in the germline before producing mutant progeny. Alternatively, transposition events that disrupt gene expression in germline stem cells can be selected in tissue culture (Fig. 1), before being used to produce transgenic animals.

Here we took advantage of the unique biology of the spermatogonium, together with state-of-the-art transposon technology, to establish an experimental pipeline for knocking out genes in the rat (Supplementary Fig. 1). We carried out transposon mutagenesis in the rat germline by transfecting primary rat spermatogonial lines with a transposon plasmid containing a gene-trap selection cassette and a helper plasmid encoding an improved, hyperactive Sleeping Beauty transposase (Supplementary Fig. 2). We derived spermatogonial lines from individual transgenic Sprague Dawley rats that express EGFP specifically in the germline (germ cell-specific (GCS) *EGFP* rats)². We clonally selected gene-trap insertions based on expression of the β -geo (*lacZ* and neomycin resistance gene) construct from the trapped allele and based on G418 resistance (Fig. 1a). We achieved a gene-trapping frequency of ~0.4% in the rat spermatogonia, which was fully dependent on transposase activity (Fig. 1b). All G418-resistant spermatogonial colonies stained positive for β -galactosidase (Fig. 1b). We defined over 150 Sleeping Beauty insertions from individually picked and expanded gene-trapped spermatogonial colonies by splinkerette PCR (Supplementary Table 1) and established 100 gene-trap events in genes distributed in 21 of the 22 rat chromosomes (Supplementary Fig. 3). Eight of these spermatogonial colonies contained disruptive exonic insertions, and 92 contained intronic gene-trap insertions (Supplementary Table 1). These results demonstrate the potential for Sleeping Beauty to disrupt a broad spectrum of genes in cultured rat spermatogonia.

To transmit stem cell genomes through the rat germline, we transplanted testes of sterile *Dazl*-deficient¹⁴ and wild-type recipient rats with mixed populations of G418-resistant colonies selected as a polyclonal library. We estimated that the generated library contained ~2,400 individual spermatogonial clonal lines with trapped genes (genetrapping frequency = 0.4% transfected cells $\times$ 20% transfection efficiency $\times$ 3×10^6 cells per transfection). Preliminary analyses of a recipient transplanted with the library showed clear development of β -galactosidase-positive spermatogonia (Fig. 1c). We analyzed genotypes of rat progeny produced by crossing recipient males with wild-type females using PCR primers designed to detect the β -geo cassette in stably integrated Sleeping Beauty transposons, and the GCS *EGFP* rat transgene as an inheritable marker for the donor spermatogonial line (Supplementary Fig. 4). We detected no donor-derived pups in litters born from a wild-type female and wild-

type recipient pair (0/56 pups born; $n = 6$ litters) using the G418-selected spermatogonia (Supplementary Table 2). However, litters born from a wild-type female and *Dazl*-deficient recipient pair yielded 100% germline transmission of the GCS *EGFP* donor cell haplotype (113/113 pups born; $n = 16$ litters), in which the β -*geo* marker was transmitted to 72% (82/113 pups born) of the total F1 progeny, averaging $69.2 \pm 29.7\%$ GCS *EGFP*, β -*geo* expressing pups per litter (mean $\pm$ s.d.; $n = 16$ litters) (Supplementary Table 2).

Because 100% of pups born were derived from donor spermatogonia (GCS *EGFP*⁺), resulting Mendelian ratios (wild type: β -*geo* = 28:72; $n = 113$ F1 pups) for transmitting Sleeping Beauty to F1 progeny signified that multiple copies of the transposon integrated into stem spermatogonia during culture (Supplementary Fig. 5). We predicted gene-trap mutations to disrupt expression of at least 30 distinct genes upon analysis of the 82 Sleeping Beauty F1 mutant rats (~35%) (Supplementary Table 3). Based on gene-specific probes for ten representative mutations (*Igsf3*, *Ube2q2*, *RGD1561493* (similar to mouse *Rgs22*), *Zmynd8* (also known as *Prkcbp1*), *RGD1562674* (similar to mouse *Ksr2*), *Arhgap26*, *Spaca6* (*LOC688452*), *Ssbp2*, *Pclo* and *Pan3*), trapped genes were stably transmitted from F1 mutant and wild-type breeders to F2 progeny, with 57 of 106 total F2 pups (~54%; $n = 10$ litters; 1 litter per mutant strain) inheriting their mutant parent's respective haplotype. Expression profiles for trapped genes were also transmitted to F2 progeny based on X-gal staining for β -*geo* expression in testes of neonatal *Pan3* mutants and in *Spaca6* mutant embryos (Fig. 1d,e).

Next, we tested the sperm-forming potential of individually picked, G418-resistant spermatogonial colonies after transfecting cultures with the Sleeping Beauty gene-trap constructs and plating at low density. We expanded five colonies that stained strongest with X-gal to $\sim 3 \times 10^6$ cells per culture. We cryopreserved portions of cells expanded from each colony and used them to define gene-trap insertions (Supplementary Table 1). We transplanted remaining portions expanded from each colony into testes of one or two *Dazl*-deficient rats per colony. At 65–75 d after transplantation, we paired recipients with wild-type female rats, and 33% of breeder pairs produced viable litters expressing the donor cell haplotype (GCS *EGFP*⁺) representing 40% of transplanted colonies (Supplementary Table 2).

Spermatogonial colony 5 (SB5), which contained a gene-trap mutation in *Slc35a3*, resulted in mutant pups from one of two transplanted recipients (19 of 19 pups born) (Supplementary Fig. 6). Spermatogonial colony SB20, which contained gene-trap mutations in *Tbc1d1*, *Ube2k* (also known as *Hip2* and *E2-25k*), *Txn13* and *RGD1560888* (similar to mouse *Cdk8*), generated mutant pups from two of two transplanted recipients (50 of 50 pups born) (Supplementary Fig. 7). Germline transmission rates of defined gene-trap mutations in each colony demonstrated clear clonal enrichment in spermatogonial colonies picked after selection in G418-containing medium (Supplementary Figs. 6 and 7 and Supplementary Discussion).

Based on semiquantitative PCR analyses using primer sets to exonic regions that flank individual introns of *Ube2k* (Fig. 2a,b), and northern blot analysis (Fig. 2c), the relative levels of *Ube2k* transcripts in adult rat brain were reduced greater than 99% in rats homozygous for the *Ube2k* gene-trap mutation. Proteins encoded by the β -*geo* reporter were expressed ubiquitously in embryonic rats that inherited the *Ube2k* gene-trap mutation (Fig. 2d). Accordingly, western blot analysis revealed that expression of the native UBE2K protein was effectively ablated in brains of adult rats homozygous for the *Ube2k* mutation (Supplementary Table 4 and Supplementary Fig. 8). Thus, rat spermatogonial stem cells can be genetically modified with Sleeping Beauty transposons in culture, clonally enriched for gene-trap mutations by selection in G418-containing medium and then used to produce knockout rats through natural breeding with recipient founders.

By this approach, we generated 35 rat lines with defined gene-trap mutations, in which greater than 50% of the trapped genes (*Abca13*, *Alms1*, *Arntl*, *Btrc*, *RGD1560888* (similar to mouse *Cdk8*), *Cdh17*, *Dlg1*, *Exoc6b*, *Fubp3*, *Grik3*, *RGD1562674* (similar to mouse *Ksr2*), *Pclo*, *Zmynd8* (also known as *Prkcbp1*), *RGD1561493* (similar to mouse *Rgs22*), *Ssbp2*, *Slc35a3*, *Tmem57*, *Tbc1d1* and *Ube2k*) were associated with biological processes linked to an assortment of human diseases (Supplementary Table 3). The generated rat models are available as a resource at http://www4.utsouthwestern.edu/hamralab/knockout_rats.htm.

This is the first report on using clonally selected germline stem cells from culture in a method to disrupt genes in the rat (or in any mammalian species other than the mouse). Use of male-sterile recipients in this method proved instrumental by eliminating competition between sperm produced from the recipient and sperm produced from transplanted, mutated stem cells¹⁴. Given the overarching popularity of the laboratory rat as a mammalian research model, an exciting prospect is to use stem cells of different epigenetic states (spermatogonia and embryonic stem cells) to broaden the spectrum of functional genomic elements that can be disrupted during mutagenesis screens. However, production of transgenic rats by injecting blastocysts with genetically manipulated cultures of embryonic stem cells remains an unsolved issue but appears feasible in the near future based on recent breakthroughs^{4,5}. Nevertheless, by combining the repertoire of contemporary transposon systems available (Sleeping Beauty, Tol2 and piggyBac)^{8,15} to push the coverage of targetable transcribed genes to saturation in spermatogonial cultures, the ability to functionally annotate genes in the rat on a genome-wide scale becomes exceedingly more practical, both technologically and economically.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Production of knockout rats using polyclonal libraries of mutant spermatogonia. **(a)** Schematic of gene-trap mutagenesis for an RNA polymerase II–transcribed gene using a Sleeping Beauty transposon. The transposon contains inverted terminal repeats (ITRs) flanking a gene-trap cassette comprising an adenoviral splice acceptor (SA) sequence followed by the promoterless *β-geo* reporter. As shown at the bottom of the diagram, insertion of the transposon into an expressed gene has a mutagenic effect by truncating the transcript through imposed splicing to an upstream exon. Such gene-trap mutations only occur if the 5' to 3' orientation of the *β-geo* cassette coincides with that of the trapped gene. **(b)** Efficiency of a hyperactive Sleeping Beauty system for gene-trap mutagenesis in cultures of rat spermatogonia based on the number

of G418-resistant, β -geo⁺ colonies selected per number of transfected cells (left). Error bars, s.e.m, n = 3 wells from a representative experiment. Image of an X-gal stained (blue), G418-resistant rat spermatogonial colony expressing β -geo from the Sleeping Beauty gene-trap vector in culture on a feeder layer of mouse embryonic fibroblasts (right). **(c)** Image of X-gal stained donor-derived colonies of differentiating spermatogonia in rat seminiferous tubules transplanted with polyclonal cultures of spermatogonial stem cells after genetic modification with Sleeping Beauty. **(d)** Expression of β -geo in neonatal testes of F2 wild-type (*Pan3*^{+/+}) and heterozygous *Pan3* Sleeping Beauty gene-trap mutant (*Pan3*^{+/-}) rat littermates produced by breeding an F1 *Pan3*^{+/-} mutant female with a wild-type male. X-gal staining (blue) is shown in testes from representative littermates. **(e)** Expression of β -geo in F2 wild-type (*Spaca6*^{+/+}) and heterozygous *Spaca6* Sleeping Beauty gene-trap (*Spaca6*^{+/-}) rat siblings at embryonic day 15.5 produced by breeding an F1 *Spaca6*^{+/-} mutant male with a wild-type female. X-gal staining (blue) is shown in representative littermates. Scale bars, 25 μ m **(b,c)**, 10 mm **(d)** and 12.5 mm **(e)**.

Figure 2.

Production of knockout rats using monoclonally enriched spermatogonial lines. **(a)** Schematic on gene-trap mutagenesis of the *Ube2k* gene using the Sleeping Beauty transposon system. Inset, genomic PCR analysis of wild-type (*Ube2k*^{+/+}), heterozygous (*Ube2k*^{+/-}) or homozygous mutant (*Ube2k*^{-/-}) genotypes of representative F2 progeny for *Ube2k* Sleeping Beauty gene-trap alleles. **(b)** Semiquantitative PCR analysis of total RNA isolated from F2 rat brains of representative genotypes produced from F1 parents heterozygous (*Ube2k*^{+/-}) for the *Ube2k* Sleeping Beauty gene-trap mutation ($n = 1$ rat per genotype). Primer pairs were design to flank introns 1, 4 and 5, or to be specific to the 5' untranslated region (UTR) of exons 1 and 3, as indicated in **a**. **(c)** Northern blot analysis of total RNA from brains of F2 rats wild-type,

heterozygous or homozygous for the *Ube2k* Sleeping Beauty gene-trap mutation ($n = 1$ rat per genotype). **(d)** X-gal staining for β -geo expression in F2 rat embryos (E14.5) of representative genotypes produced from F1 parents heterozygous for the *Ube2k* Sleeping Beauty genetrapped mutation. Scale bar, 1 cm.